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Cell culture, transfections and immunofluorescence

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Updated date: May 12, 2021

An abbreviated version of this protocol was published in eLIFE in Oct 2014

A clathrin coat assembly role for the muniscin protein central linker revealed by TALEN-mediated gene editing

DOI: 10.7554/eLife.04137

Detailed protocol

Cell culture, transfections and immunofluorescence

Cell culture

1. Grow HeLa SS6, genome edited FCHO2 functionally-null HeLa clone #64, genome edited FCHO1 + FCHO2 functionally-null HeLa SS6 clone #64/1.E (1.E cells), the neuronal SH-SY5Y and the human breast cancer MCF-7 cells in DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine at 37°C in an atmosphere of 5% CO_2.
2. Grow HeLa SS6 cells, stably transfected with a plasmid encoding a YFP-tagged β2 subunit of AP-2, in DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine containing 0.5 mg/ml G418 at 37°C in 5% CO2.
3. Grow human myleogenous leukemia cell line, K562 in suspension in RPMI media supplemented with 5% fetal calf serum and 2 mM L-glutamine at 37°C in 5% CO_2.

Transfections

1. Seed trypsinized cells into 35 mm cell culture dishes containing 12 mm #1 round glass coverslips.
2. Grow cells in DMEM supplemented with 10% fetal calf serum and 2 mM L-glutamine (complete media) overnight at 37°C in 5% CO2
3. Next day, cells are ready for transfection.
4. Make transfection mix by combining 0.5-1 µg plasmid DNA, 6 µl Lipofectamine 2000 and 375 µl Opti-MEM I Reduced Serum medium
5. Incubate the transfection mix at room temperature for 40 minutes.
6. Remove the old media in dishes and add 1.5 ml fresh complete media.
7. Add transfection mix dropwise to cells and gently swirl the dishes.
8. Grow cells at 37^0C, 5% CO_2 for another 18-24 hours and then use for immunofluorescence.
9. For siRNA experiments, transfect cells in two rounds using 10 µl (200 pmoles of each pool) of siRNA along with 5 µl Oligofectamine and 185 µl Opti-MEM.
10. After transfection, incubate cells in serum free media for 4-6 hours
11. Then, add 500 µl Opti-MEM I containing 30% FBS to cells and incubate for another 18-24 hours at 37^0C in 5% CO2 atmosphere.
12. Next day, trypsinize cells and plate into new dish containing coverslips for second round of transfections as in step-9.

Immunofluorescence

Protocol #1:

1. Rinse cells grown on coverslips three times with PBS and fix at room temperature for 20 minutes with 2% paraformaldehyde (PFA) in PBS.
2. Wash three times in ice cold PBS.
3. Incubate cells at room temperature for 30 minutes in blocking/permeabilization buffer (10% normal goat serum, 0.2% saponin in PBS).
4. Remove blocking/ permeabilization solution and incubate cells at room temperature for 60 minutes in antibody dilution buffer (10% normal goat serum, 0.05% saponin in PBS) containing appropriate concentration of primary antibody.
5. Wash three times in ice cold PBS.
6. Incubate cells in Alexafluor-conjugated secondary antibody diluted in antibody dilution buffer for 60 minutes at room temperature in dark.
7. Clarify antibody solutions by centrifuging at 15,000 x g_{max} for 10 minutes before adding into permeabilized cells.
8. Wash three times in ice cold PBS.
9. Incubate cells with 1 µg/ml Hoechst 33342 DNA dye made in PBS for 10 minutes at room temperature
10. Wash two times in ice cold PBS
11. Mount coverslips on glass slides in Cytoseal.
12. Air dry samples and perform microscopy.

Protocol #2 (modified IF):

1. Rinse cells twice with PBS and then fix them for at least 10 minutes at room temperature with 4 % PFA in PBS, pH- 8.0.
2. Wash cells twice with PBS and quench 10 minutes at room temperature with 75 mM ammonium chloride and 20 mM glycine dissolved in PBS, pH- 8.0.
3. Wash thrice with PBS and block/ permeabilize 10 minutes at 37^0C with goat serum (5%) and Triton-X-100 (0.1%) diluted in PBS-fish skin gelatin-saponin (PBS-FSG-saponin: 7 mg/ml fish skin gelatin, 0.05% saponin in PBS, pH- 8.0).
4. Incubate with primary antibody diluted in PBS-FSG-saponin for 60 minutes at 37^0C. Remember to spin down antibody solutions before use for 5 min, max speed in a bench top centrifuge.
5. Wash samples three times with PBS-FSG-saponin.
6. Incubate with Alexafluor-conjugated secondary antibody diluted in PBS-FSG-saponin for 60 minutes at 37^0C.
7. Wash three times with PBS-FSG-saponin and three times with PBS alone.
8. To reduce background, incubate samples with 0.1% Triton-X-100, dissolved in PBS, for 5 minutes at room temperature, followed by a 5 minute wash in PBS alone.
9. Post-fix the cells in 4% PFA dissolved in 100 mM sodium cacodylate, pH 7.4 for 30 minutes at room temperature.
10. Wash the cells twice with PBS, pH- 8.0 and quench as in step-2.
11. Wash the cells three times with PBS, pH- 8.0.
12. Incubate cells with 1 µg/ml Hoechst 33342 DNA dye, made in PBS, for 10 minutes at room temperature.
13. Wash cells twice with PBS, pH- 8.0
14. Mount coverslips on glass slides in Cytoseal.
15. Air dry samples and perform microscopy.

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Umasankar, P. (2021). Cell culture, transfections and immunofluorescence. Bio-protocol Preprint. bio-protocol.org/prep1081.
2. Umasankar, P. K., Ma, L., Thieman, J. R., Jha, A., Doray, B., Watkins, S. C. and Traub, L. M.(2014). A clathrin coat assembly role for the

muniscin protein central linker revealed by TALEN-mediated gene editing. eLIFE. DOI: 10.7554/eLife.04137